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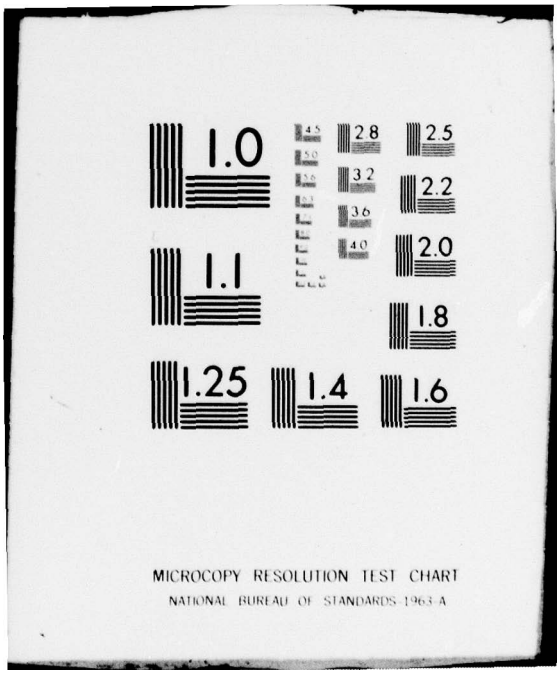
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In Vitro Studies on the Interaction of Rickettsia and Macrophages

I. Effect of Ultraviolet Light on Coxiella burnetii Inactivation and Macrophage Enzymes

JAMES S. LITTLE,* RICHARD A. KISHIMOTO,[1] AND PETER G. CANONICO

United States Army Medical Research Institute of Infectious Diseases
Fort Detrick, Frederick, Maryland 21701

Running title: UV-INACTIVATED C. BURNETII/MACROPHAGE ENZYMES

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

[1]Present address: SGRD-OP (LTC Kishimoto), Fort Detrick, Md 21701

Telephone no. of Dr. Little: (301) 663-7341

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The inactivation of Coxiella burnetii in suspension or in cultures of guinea pig peritoneal macrophages by ultraviolet (UV) light was studied. The effect of UV treatment on the activity of macrophage organelle marker enzymes and their subsequent equilibration in linear sucrose gradients was also determined. It was shown that UV treatment of 600 $\mu W/cm^2$ for 15 sec at a distance of 10 cm inactivated C. burnetii, either in suspension (10^8 organisms/ml) or within guinea pig peritoneal macrophages. Similar UV treatment had little effect on the activity or equilibration of macrophage organelle marker enzymes in linear sucrose gradients. However, longer exposure caused considerable inactivation of these enzymes.

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ABSTRACT

The inactivation of Coxiella burnetii in suspension or in cultures of guinea pig peritoneal macrophages by ultraviolet (UV) light was studied. The effect of UV treatment on the activity of macrophage organelle marker enzymes and their subsequent equilibration in linear sucrose gradients was also determined. It was shown that UV treatment of 600 ~~μW/cm²~~ erg/cm² for 15 sec at a distance of 10 cm inactivated C. burnetti, either in suspension (10^8 organisms/ml) or within guinea pig peritoneal macrophages. Similar UV treatment had little effect on the activity or equilibration of macrophage organelle marker enzymes in linear sucrose gradients. However, longer exposure caused considerable inactivation of these enzymes.

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While designing experiments to determine the intracellular fate of Coxiella burnetii in guinea pig peritoneal macrophages, it was necessary as an safety precaution to inactivate ingested rickettsiae without significantly inactivating macrophage enzymes. These enzymes would subsequently be used as markers for determining the distribution of cellular organelles on linear sucrose gradients after homogenization and fractionation of macrophages containing ingested rickettsiae. Therefore, inactivation of the rickettsiae without inhibition of macrophage organelle marker enzymes was a prerequisite. Chemical fixation, alcohol denaturation, heat, and a number of other conventional methods of inactivation were known to affect adversely enzyme activity. Previous studies have shown that rickettsiae in suspension could be inactivated by exposure to ultraviolet (UV) light (1, 14). However, the effect of UV light on rickettsiae growing in macrophages and on the activity of macrophage organelle marker enzymes was unknown. This paper describes a method for the inactivation of C. burnetii in suspension or in guinea pig peritoneal macrophages without considerably altering the activity of several marker enzymes for macrophage subcellular components.

MATERIALS AND METHODS

Chemicals. Methyl-[^{3}H]thymidine (47 Ci/mmol) and [^{3}H(G)]adenosine-5'-monophosphate (13.5 Ci/mmol) were obtained from New England Nuclear (Boston, Mass.). Scintisol complete was obtained from Isolab, Inc. (Akron, Ohio). Ultra-pure, ribonuclease-free sucrose was obtained from Schwarz/Mann (Mountain View Avenue, Orangeburg, N.Y.). The 4-methylumbelliferyl substrates used in the biochemical assays were purchased from Koch-Light Laboratories, Ltd. (Colnbrook, Buckinghamshire, England). All other chemicals were of analytical grade when available and were obtained from commercial sources.

Animals. Outbred male Hartley strain guinea pigs, weighing approximately 350 to 450 g, and outbred white mice [Tac:(SW)fBr] weighing 18 to 22 g were obtained from Buckberg Lab Animals (Tompkins Cove, N.Y.).

Culture and labeling of rickettsial stock suspension. The third egg passage of the Henzerling strain of *C. burnetii* in phase I and the 88th egg passage of the phase II Nine-Mile strain were grown in chicken embryo cells as previously described (10). Nine days after inoculation the medium was removed; 50 ml of fresh medium containing 10 uCi methyl-[^{3}H] thymidine were added to each roller bottle. Rickettsiae were harvested 24 h later as previously described (10). Samples were pooled and rickettsial counts determined by the method of Silberman and Fiset (16).

Preparation of guinea pig peritoneal macrophages. Peritoneal exudate cells were collected 4 days after guinea pigs were injected intraperitoneally (i.p.) with 20 ml of a 1.5% sodium caseinate solution. Cells were harvested and processed according to the method of David et al. (16). Approximately 5 x 10^6 macrophages were dispensed into petri

dishes (15 cm in diameter; Falcon Plastics, Oxnard, Calif.) and incubated at 37°C for 2 h in a humid atmosphere of air containing 5% CO_2. Nonadherent cells were removed by two washes with Hanks' balanced salt solution (HBSS) and then 5 ml of fresh E-199 medium were added.

Infection of macrophages. Rickettsial suspensions containing approximately 10^8 organisms/ml of either phase I or II C. burnetii were incubated for 30 min at 37°C in Earle 199 medium containing 10% heat-inactivated anti-phase I or II C. burnetii guinea pig serum (1:1,000 microagglutination titer). Serum-treated rickettsiae were then added to macrophage cultures in a ratio of 100 rickettsiae/macrophage and incubated at 37°C for 60 min. The inoculum was removed and macrophage cultures were washed three times with 20 ml HBSS.

Ultraviolet light irradiation. Control and infected macrophage cultures, washed two times with 10 ml HBSS were overlaid with 10 ml of HBSS so that the depth of the overlay was approximately 1 mm. The cultures were then placed on ice and exposed to UV light (600 $\mu W/cm^2$, Sylvania No. G15T8 germicidal tube) at a distance of 10 cm for up to 10 min. After exposure to UV, macrophages were scraped off the petri dish with a rubber-tipped scraper. The suspension media was collected; an additional 10 ml of HBSS were added to the culture dishes and the plates were scraped again. The macrophages contained in the pooled suspension media were then either assayed directly for marker enzymes or homogenized and fractionated as described below.

Three ml of rickettsial suspension containing 1 x 10^8 organisms/ml of phase I or II C. burnetii were dispensed into petri dishes (8 cm in diameter) which resulted in suspensions approximately 1 mm in depth.

The suspensions were then exposed to UV light for 15 sec at a distance of 10 cm.

Titration of rickettsiae. Various dilutions of rickettsial suspensions and infected macrophages were inoculated i.p. into four white mice for each dilution point. The animals were bled 21 days later; sera were checked for specific antibodies by indirect immunofluorescence (4).

Homogenization of guinea pig peritoneal macrophages. Irradiated or control macrophage suspensions were sedimented at 480x g for 5 min in a Sorvall RC3-B refrigerated centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The cellular pellet was homogenized in 0.25 M sucrose containing 1 mM ethyleneglycol-bis (β-amino-ethyl ether)N, N'-tetra-acetic acid (EGTA) and 3 mM imidazole-HCl buffer (pH 7.5) with a syringe by 7 passages through a 12-inch long, 25-gauge cannula (5). The nuclei and unbroken cells were sedimented by differential centrifugation at 480x g for 5 min. The supernatant (designated as cytoplasmic extract) was saved and the pellet rehomogenized and recentrifuged as described above. This process was repeated four times; the cytoplasmic extracts from the centrifugations were combined.

Fractionation of cytoplasmic extracts. An aliquot of the pooled cytoplasmic extract was layered on a sucrose gradient containing 3 mM imidazole-HCl buffer (pH 7.5) and extending linearly with respect to volume from a density of 1.10 to 1.25. The gradient contained in a Beckman Quickseal tube rested on a 5-ml cushion of 1.3 M buffered sucrose. Centrifugation was in a Beckman V50 rotor for 90 min at 40,000 rpm in a Beckman L2-65 B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Following centrifugation, 12 aliquots

of approximately equal volume were pumped from the centrifuge tube into tared tubes. The fractions, maintained at 4°C, were weighed and their contents mixed. The density of each fraction was determined on a Bausch and Lomb Abbe III refractometer. Each fraction was then assayed for enzymatic activity as described previously (5).

Determination of radioactivity. For determination of radioactivity 0.2 ml of each fraction was mixed with 0.8 ml H_2O. To this, 20 ml of Scintisol complete was added. Samples were counted in a Mark III liquid scintillation spectrophotometer (Searle, Des Plaines, Ill.). Counting efficiency was approximately 41% and differential quench from vial to vial was not encountered.

Presentation of results. The distribution of phase I and II C. burnetii in linear sucrose gradients and the distribution patterns of enzymes after fractionation of cytoplasmic extracts in linear sucrose gradients are presented in the form of histograms constructed as previously described (2, 3, 7, 11).

RESULTS

Ultraviolet light irradiation. Approximately 10^8 viable phase I or II C. burnetii in suspension were exposed to UV light for 15 sec. Following treatment, various dilutions (10°-10^{-8}) titrated i.p. in white mice failed to elicit detectable serum antibodies at 21 days. Similar results were obtained when macrophages were infected with 10^8 viable phase I or II C. burnetii/ml. UV treated and titrated in the same manner. Previous studies have shown that one phase I organism or one phase II (EP 88) can infect and seroconvert white mice (unpublished data). These results suggest that exposure to UV light for 15 sec inactivates phase I and II C. burnetii both in suspension and in macrophage cultures.

Effect of UV treatment on the activity of macrophage organelle marker enzymes. The activity of macrophage organelle marker enzymes was considerably decreased when macrophage cultures were exposed to UV light for periods up to 10 min (Fig. 1). The range of inhibition at 10 min of UV exposure was from 51% for malate dehydrogenase to 100% for alkaline α-glucosidase and N-acetyl-β-glucosaminidase (Fig. 1). However, after 15 sec of exposure, inhibition did not exceed 35% (5'-nucleotidase, Fig. 1); in fact, the activity of both lysosomal enzyme markers (α-D-galactosidase and N-acetyl-β-glucosaminidase) increased by 50 and 20% respectively (Fig. 1). The reasons for activation of lysosomal enzymes by UV light of short duration is not apparent. It does not appear to be due to the unmasking of structure-linked latency exhibited by lysosomes (8) since both enzyme assays contained detergent concentration sufficient to unmask latent activity.

Fractionation of cytoplasmic extracts. Exposure of macrophages to UV light for 15 sec does not appear to alter the buoyant properties of macrophage organelles. The distribution and mean equilibrium density of marker enzymes following centrifugal equilibrium on sucrose gradients remained essentially unchanged after UV exposure (Fig. 2).

Equilibration of C. burnetii on linear sucrose gradients and determination of enzyme activity. Radiolabeled phase I and II C. burnetii, irradiated for 15 sec, were layered on sucrose gradients and centrifuged to equilibrium. Phase I had an equilibrium density in the alkaline sucrose of 1.24 (Fig. 3), a value which is in agreement with previous reports (17, 18). Phase II was found to have an equilibrium density of 1.23. When gradient fractions were assayed for malate dehydrogenase, 5'-nucleotidase, alkaline α-glucosidase, α-D-galactosidase, alkaline phosphatase, and N-acetyl-β-glucosaminidase, no detectable quantities of these macrophage marker enzyme activities were found in either the phase I or II C. burnetii gradients.

DISCUSSION

The present study shows that phase I and II C. burnetii either in suspension or growing within cultured guinea pig peritoneal macrophages are inactivated by brief exposure to UV light. Treatment of C. burnetii with UV decreases the risk of individuals working with this highly virulent organism. Since certain procedures required for subsequent studies on the interaction of C. burnetii with macrophages could cause aerosolization, working with an inactivated organism was desirable. It must be noted, however, that photoreactivation of UV-inactivated organisms can take place (15). In our studies, once organisms were UV-treated,

exposure to light was kept to a minimum by wrapping tubes containing C. burnetii in aluminum foil and storing in the cold. This precaution should help minimize photoreactivation, but reactivation by exposure to light was not tested.

We have shown that exposure of macrophage cultures to UV light for 15 sec does not appreciably inactivate marker enzymes for macrophage organelles or their subsequent equilibration on linear sucrose gradients. These results suggest that UV treatment does not adversely affect the conformation of these proteins or alter the permeability to sucrose of lysosomes, microsomes, or mitochondria. Since these enzymes retain most of their activity after UV treatment, they may be used confidently as markers for the localization of cellular constituents on linear sucrose gradients following fractionation of macrophage homogenates. It is now possible to determine the intracellular distribution and fate of C. burnetii in guinea pig peritoneal macrophages using analytical methods of subcellular fractionation.

Finally, it has been shown that rickettsiae contain a number of different enzymes (12, 13). Our results suggest that neither phase I or II C. burnetii contain detectable amounts of the enzymes assayed in these experiments. However, it must be noted that these preparations were UV-treated prior to assay. The effect of this UV treatment on the rickettsial enzymes is not known. The absence in phase I and II C. burnetii of detectable quantities of enzymes chosen as markers for macrophage organelles greatly simplifies and facilitates fractionation studies. It eliminates the requirement for correcting macrophage enzyme activities due to rickettsiae.

ACKNOWLEDGMENTS

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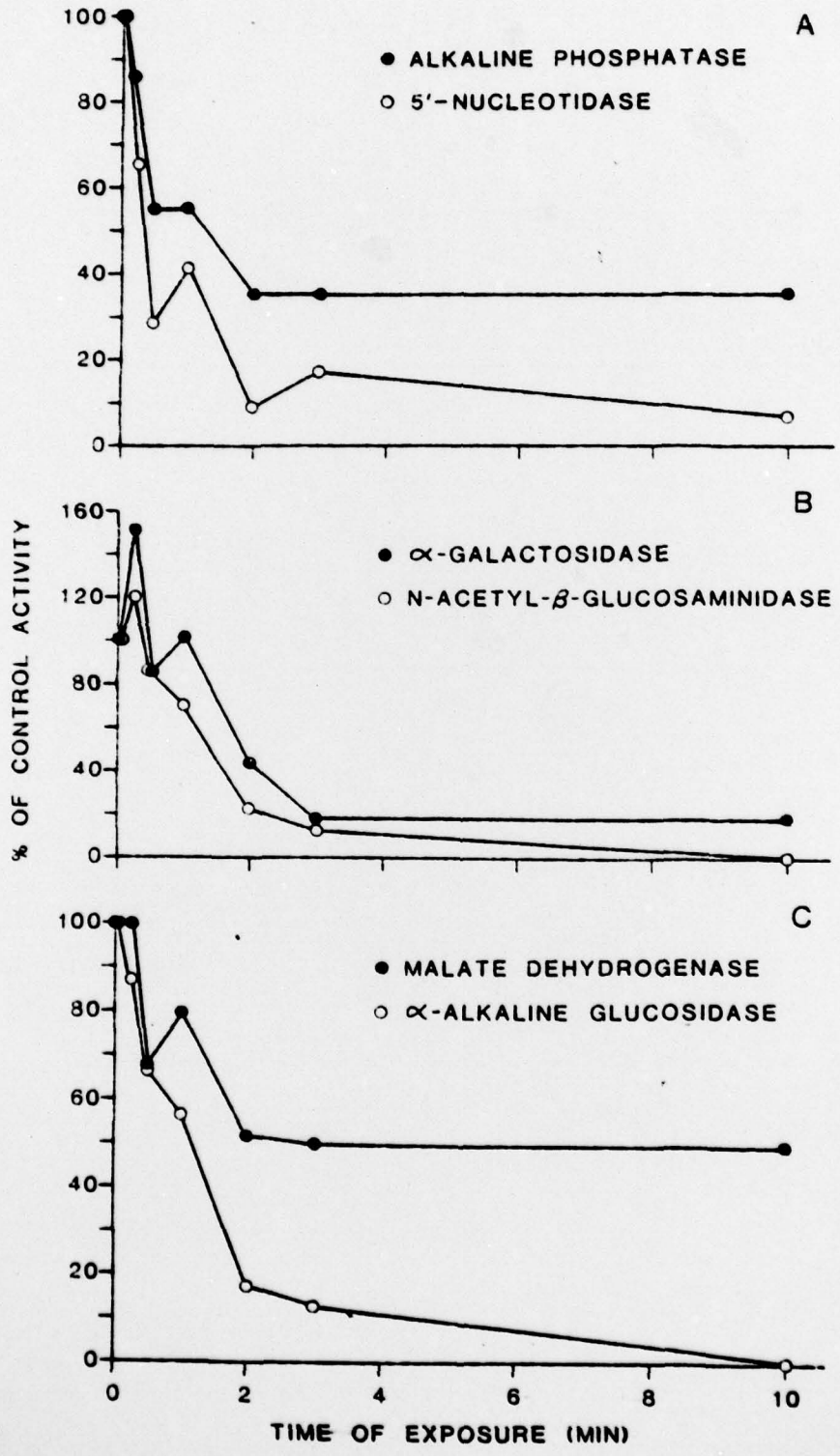
LEGENDS TO FIGURES

Fig. 1. Effect of UV treatment on activity of macrophage enzymes. UV treatment and enzyme assays were as described in Materials and Methods. Results are those of one representative experiment.

Fig. 2. Distribution profiles of constituents from cytoplasmic extract of control (——) or UV-treated (– – –) guinea pig peritoneal macrophages. Macrophages were treated with UV light for 15 sec as described in Materials and Methods. Results are presented in the form of normalized and averaged frequency histograms. The density scale, divided into 15 normalized fractions of identical density increment, extends from 1.10 to 1.25. The frequency represents $\Delta Q/(\Sigma Q \Delta \rho)$, where ΔQ is the amount of constituent present within the section, and ΣQ the sum of the amounts found in all the subfractions. The surface area of each histogram bar then gives the fractional amount of constituent present within each normalized fraction. $\Delta \rho$ is equal to 0.0113 density units. Distribution profiles are flanked on either side by blocks arbitrarily constructed over the density spans 1.06 to 1.10 and 1.25 to 1.30 and refer to material recovered above and below the linear limits of the gradient. The total area of each histogram is then equal to 1. Diagrams show averages of results with standard errors of the mean. The numbers in the upper right hand corner of each figure represent the recovery of enzyme activity.

Fig. 3. Equilibration of radiolabeled phase I (A) and II (B) C. burnetii on sucrose gradients. Results are expressed as described in the Legend to Fig. 2. The numbers in the upper right hand corner represent the recovery of the label.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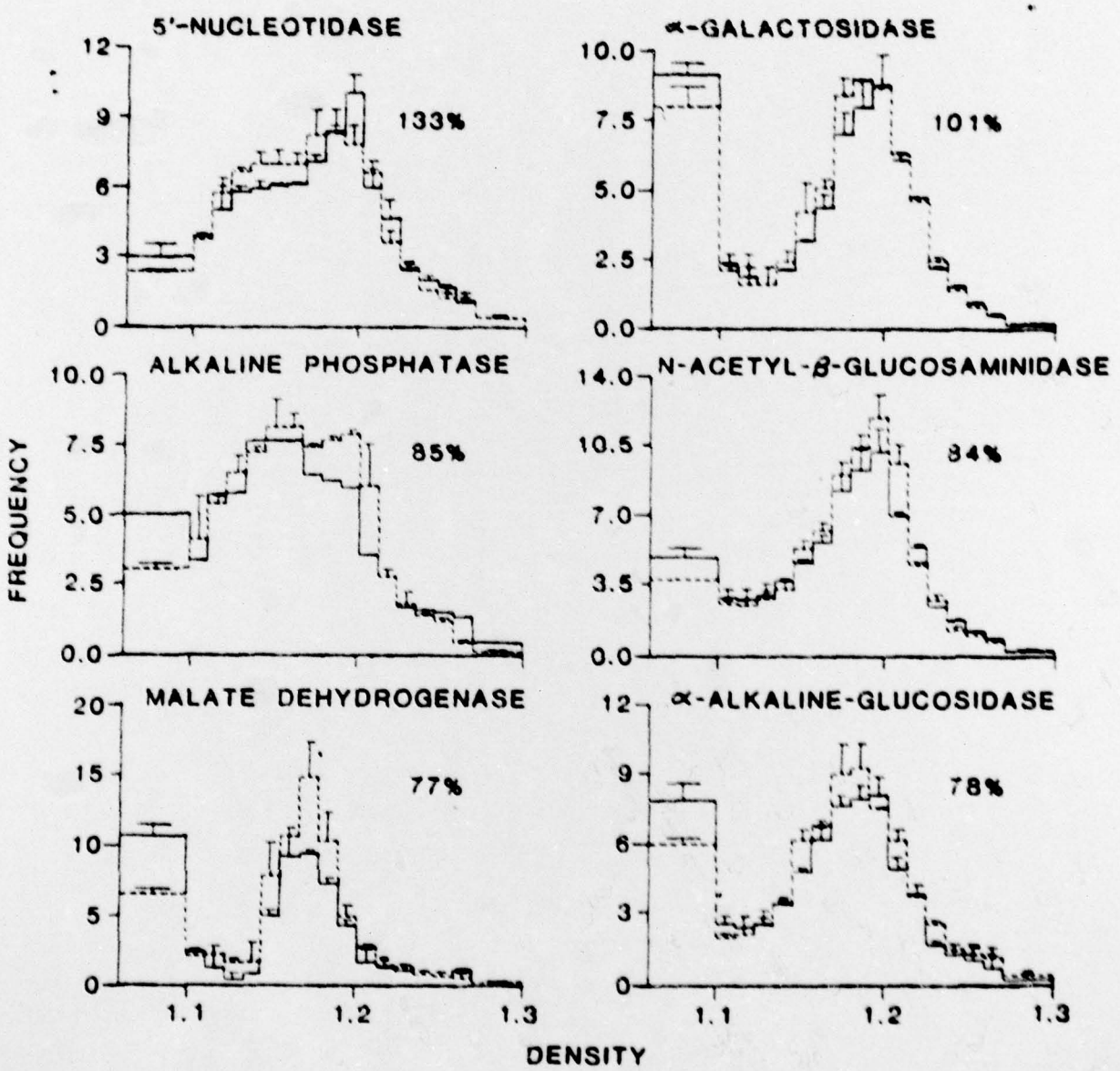
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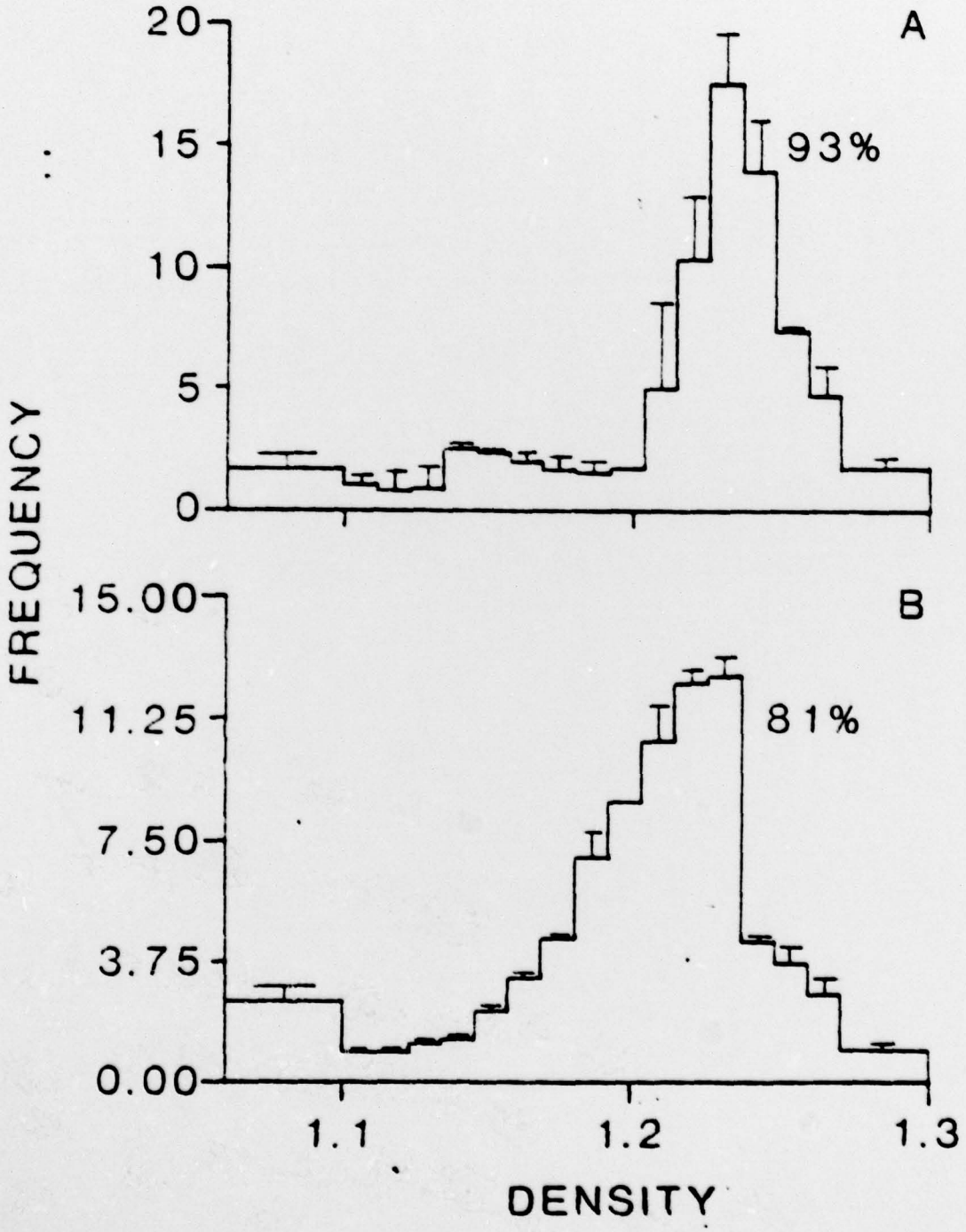
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